II.7—IMPROVED METHOD FOR MEASURING BIODEGRADATION RATES OF HYDROCARBONS IN NATURAL WATER SAMPLES

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INTRODUCTION

An improved method is described for the determination of biodegradation kinetics of fossil-derived hydrocarbons in natural water samples. This method is a modification of protocols in which ^{14}C-labeled substrates are incubated in natural samples, and $^{14}CO_2$ is measured to determine total mineralization. As with other methods, the described protocol allows for the measurement of utilization and uptake. However, it adds the ability to measure the remaining substrate and polar metabolic intermediates formed during biodegradation. The fate of the isotopic tracer is determined by mass balance. Water samples are incubated with tracer amounts of ^{14}C-labeled substrates. After incubation, $^{14}CO_2$ is trapped in NaOH-soaked filter papers. Cells are collected on 0.22 μm filters, and the filtrate is extracted through C_{18} solid-phase extraction columns and the effluent then collected. These fractions are measured, respectively, as CO_2 (mineralization), bacterial uptake (incorporation and adsorption), unused substrate (C_{18}-extractable), and nonextractable remainder (polar intermediates and residual nonextractable substrate—depending on extraction efficiency). Total activity is calculated as the sum of all recovered isotopes, and each value is expressed as a fraction of this total. Killed control fractions are also calculated as a percent of total activity, and these fractions are subtracted from the experimental total activity to yield activity values. Isotope dilution is calculated from an ambient concentration of the tested substrate as determined by gas chromatography/mass spectroscopy (GC/MS) analysis.

The advantages of the described method are increased accuracy (particularly with respect to replicate pipetting), reduced radioactive waste volume, the ability to determine absorption of hydrophobic substrates to glass surfaces, and the amount of polar intermediates produced during biodegradation. The measurement of the biodegradation rate for hydrocarbons is key to determining the suitability of bioremediation as a treatment strategy for cleanup of the thousands of contaminated sites within the United States and abroad. The method was developed for natural water samples but could be used in pure cultures to accurately determine biodegradation kinetics during time-course experiments. Data from benzene, toluene, ethylbenzene, xylenexs (BTEX) and creosote-impacted field sites are presented.

A number of methods have been used to assess the biodegradation of various substrates in environmental settings. Radio labeled tracers have become favorable since they are generally available, can often be added in "tracer" amounts, and in the case of ^{14}C-labeled substrates, total biodegradation is straightforward to assess (by measuring the production of $^{14}CO_2$) [3]. The exploitation of this means of investigation has produced numerous publications outlining its use for various substrates, such as amino acids [5], carbohydrates [2], and pollutants [9]. In addition to using radio-labeled pollutants, many investigators have relied on the analysis of residual substrate to determine growth rates.

This chapter describes an improved method for determinating microbial mineralization kinetics of organic pollutants. It is optimized for aromatic compounds but should be suitable for other hydrophobic

molecules. The advantages of this improved method are the ability to calculate total mineralization (based on recovery of $^{14}CO_2$) as well as polar metabolic intermediate production per-unit time. The method relies on collecting each metabolically relevant isotope fraction: $^{14}CO_2$, cellularly incorporated (and/or sorbed) balance for the fate of radio isotope is straightforward. Because fractions are based on this mass balance approach, inconsistencies in substrate addition and recovery can be taken into account. In addition, this technique allows the direct determination of "bottle effects." This provides a more definitive metabolic picture of the microbial consortium or culture and allows the investigator to accurately describe the fate of the compound(s) of interest.

MATERIALS AND METHODS

Reagents

Methanol was of HPLC grade and was purchased from Baker Chemical Corp. Radio-labeled substrates consisted of UL-^{14}C-toluene (52.3 mCi/mmol), UL-^{14}C-benzene (48.1 mCi/mmol), UL-^{14}C-napthalene (49.8 mCi/mmol), phenanthrene-9-^{14}C (13.3 mCi/mmol), and $NaH^{14}CO_3$ (30.8 mCi/mmol) and were purchased from Sigma Chemical Co. All growth substrates were dissolved in HPLC-grade methanol to give approximately 100,000 DPM/μL (except UL-^{14}C-napthalene, which was shipped from the supplier in methanol solution). $NaH^{14}CO_3$ (solid) was diluted to approximately 100,000 dpm/μL with sterile MilliQ water. Optima Gold (Packard Instruments) served as scintillation cocktail for all experiments. Buffered formalin (Fisher Scientific) was used for killed controls (1% final) and cell preservation (10% final).

Sampling Locations

Two sample locations were selected to obtain hydrocarbon-impacted water. One location is the Naval Construction Battalion Command (NCBC), Port Hueneme, California, which is the site of a fuel-line leak estimated to have contaminated an unconfined, semiperched aquifer with approximately 10,800 gal of gasoline. The sampling area was adjacent to the Navy Exchange gasoline center. At this site, the water table is about 8-10 ft below the ground surface (90% of which is covered with asphalt). Twenty recently introduced sampling wells were used to draw water samples (via vacuum). In addition, four groundwater circulation wells (GCWs) were sampled during the process of developing this method (see Chapter III.1, Heard).

The other location was a former wood-treatment facility (Cabot/Koppers Superfund Site) in Gainesville, Florida. The unconsolidated, unconfined aquifer is impacted with creosote constituents, primarily polycyclic aromatic hydrocarbons (PAHs). The groundwater is approximately 3-6 ft below the ground surface, and there are indications of residual nonaqueous phase liquid (NAPL) in the unsaturated zone and both NAPL and dense nonaqueous phase liquid (DNAPL) in the saturated zone. The study area portion of this site has 12 groundwater monitoring wells, as well as a GCW unit from which samples were taken for method development.

One additional site to obtain "clean" samples was used for optimizating pH, determining recovery efficiencies, and validating results. Water samples were taken from Four Mile Run creek in the vicinity of Arlington, Virginia. This creek runs through a suburban area outside Washington, D.C. and while not pristine, is sufficiently uncontaminated to be stocked for recreational fishing. Negligible quantities of BTEX and/or PAHs were assumed.

Experimental Design

Samples were collected via vacuum from groundwater wells, and 25 mL subsamples were placed in 50 mL amber serum bottles fitted with rubber sleeve septa (Whatman). Six subsamples were taken for each well and contaminant substrate used. Filter paper strips (Fisher P8) were cut into approximately 1 cm × 10 cm strips and "accordion" folded and then placed in 12-mm test tube caps (Fisher) with a loop of polypropylene line attached. Filter papers were soaked with 150 μL of 1 N NaOH and suspended in the headspace of incubating serum bottles. Three of the six replicates were immediately amended with 250 μL of formalin (1% final). After 3-10 h of incubation (depending on sample site), the treatment vials were amended to 1% formalin (as the controls at $t = 0$), and 100 μL of 2 N H_2SO_4 was added via syringe to each serum vial (treatment and control). The vials were placed on a rotary shaker (100 rpm) overnight to allow CO_2 evolution and trapping. After shaking, the filter papers were removed from their suspended cup and placed into scintillation vials. The remaining liquid sample was vacuum processed through the following series (using a Supelco solid phase extraction (SPE) manifold). A 20 mL syringe (for sample holding) was fitted onto a Gelman in-line filter holder containing a Millipore 0.22 μm-type GS filter. This filter holder was fitted through a cap adapter onto a preconditioned Fisher Prep-Sep C_{18} solid-phase extraction SPE column (for extraction of unused substrates). Column effluent was collected in a 40 mL vial within the vacuum manifold. After vacuuming was complete, the filter was removed and placed in a scintillation vial, the SPE column was eluted with 1 mL of methanol into a scintillation vial, and 1 mL of the collected effluent was transferred to a scintillation vial. Five mL of cocktail were then added to each vial, and the vials were transferred to a Packard Tricarb 1500 scintillation counter for 5-min radioassay. Each of the six replicates (three treatment, three controls) produced four scintillation vials containing fractions as follows: (1) accordion filter papers = liberated $^{14}CO_2$, (2) filters = bacterial cells and particles, (3) extract = unutilized dissolved substrate, (4) effluent water = polar intermediates + unextracted dissolved substrate (Fig. 1). An efficiency control for CO_2 recovery was similarly treated

[redacted]

isotope. Then each separate measurement was expressed as a percent of the total. Replicates from control and treatment samples were then averaged to arrive at a mean percent of total recovered isotope per sample. The control values were subtracted from the treatment values by the following formula:

$$(\textit{treatment fraction}) \times (\textit{treatment total recovered}) - (\textit{control fraction}) \times (\textit{treatment total recovered}) = \textit{fraction DPM}.$$

The contribution of isotope dilution was calculated by dividing the control-corrected DPM values by the ratio of the added substrate (based on specific activity) to the ambient contaminant concentration. These DPM values were then converted to μg of ^{14}C-labeled substrate based on its specific activity. The fraction of isotope "lost" (presumably to the sides of the incubation vessels or from volatilization) was calculated by dividing the total recovered DPM by the total DPM added. Finally, the μg/L/h was calculated by factoring incubation time and incubation volume. Values for CO_2 and uptake/incorporation were combined to give "traditional" utilization, and since appreciable quantities of label routinely showed up in the flow-through fraction, total utilization was calculated as the sum of respiration (CO_2 fraction), uptake (cellular fraction), and intermediate production (flowthrough fraction). Table 1 provides a sample of the calculations made. Statistical calculations were made by addition of percent error for products and quotients of measurements (calculated as standard deviation percent).

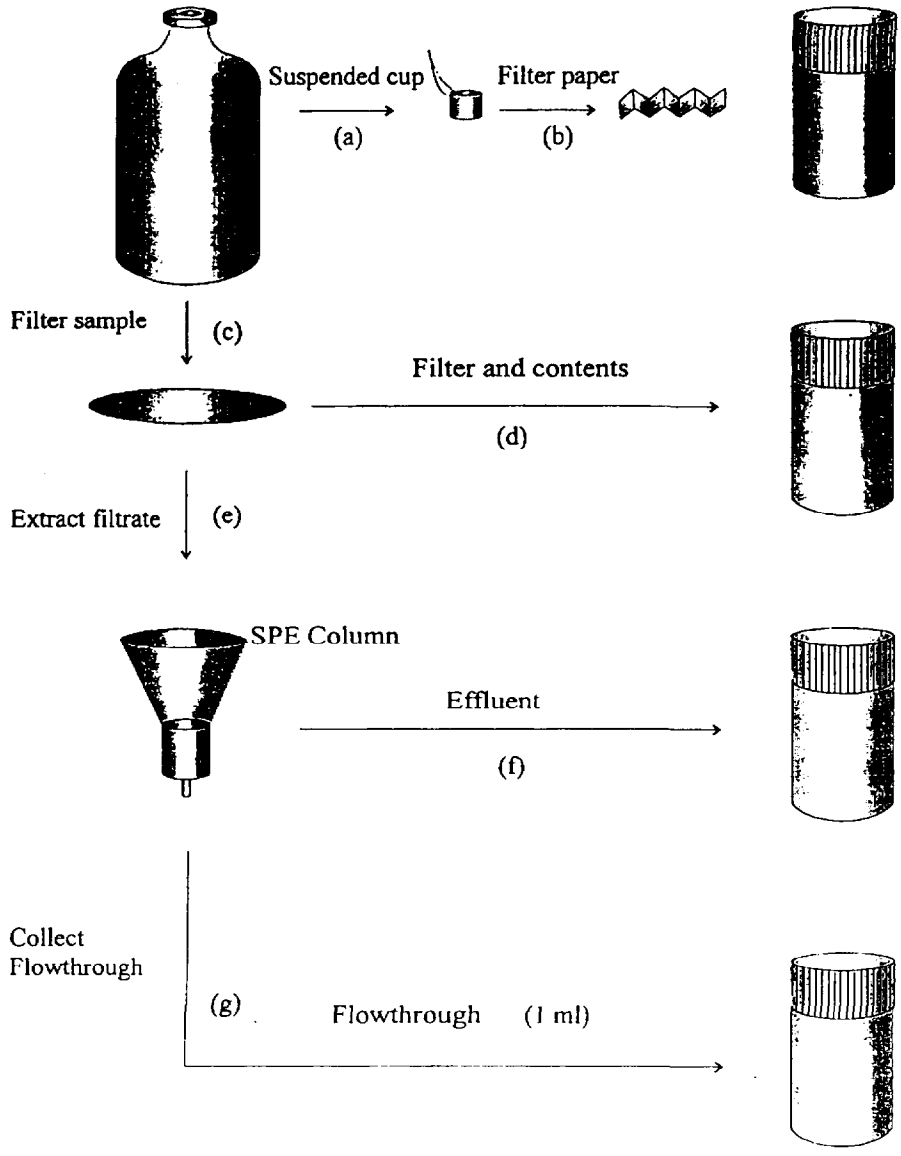

Fig. 1 — Schematic for sample processing

Table 1 — Sample Calculation Table for GCW at Port Hueneme Site
(benzene is the test substrate)

Sample	DPM	Total DPM	%	Mean μ (%)	Adj	Inc. Time (h)	Added ($\mu g \cdot L^{-1}$)	Recov. ($\mu g \cdot L^{-1}$)	Ambient ($\mu g \cdot L^{-1}$)	Util. ($\mu g \cdot L^{-1} \cdot h^{-1}$)	Util. ($\mu g \cdot L^{-1} \cdot h^{-1}$)
UVB1 Benz			7.20			9.20	6.12	3.13	81.9		
CO_2	7381		8.76								
CO_2	10138		7.55								
CO_2	7766		21.5	7.84	7923					0.0373 ± 18%	
Cells	22056		23.1								
Cells	26688		24.9								Resp + uptake
Cells	25579		59.9	23.1	13766					0.0451 ± 12%	0.0824 ± 18%
Extract	61419		59.2								
Extract	68533		55.3								
Extract	56910		11.4	58.1	−32814					−0.0626 ± 7%	
Flowthrough	468	102552	9.00								
Flowthrough	415	115753	12.3								"Total"
Flowthrough	504	102851		10.9	11638					0.0823 ± 15%	0.160 ± 18%
UVB1 Benz Kill						9.20	6.12	3.71	81.9		
CO_2	770		0.56								
CO_2	438		0.39								
CO_2	463		0.35	0.43							
Cells	15993		11.7								
Cells	10552		9.35	10.3							
Cells	12865		9.78								
Extract	118901		87.2								
Extract	101115		89.6	88.8							
Extract	117913		89.6								
Flowthrough	29	136385	0.53								
Flowthrough	30	112856	0.67	0.48							
Flowthrough	13	131562	0.24								

Determination of Ambient Concentrations for Tested Substrates

Growth substrates tested—benzene, toluene or naphthalene, and phenanthrene—were determined by the Environmental Protection Agency's (EPA) SW-846 method 8270A and EPA SW-846 method 8260A, respectively.

Estimation of Bacterial Population Size

Subsamples of collected groundwater were amended to 2% with buffered formalin and stored at 4° C until processed for acridine orange direct counts [6].

RESULTS

Mineralization rates were extremely low in the Gainesville site samples. Most samples showed no detectable mineralization, with only 4 of 14 samples in the positive range (Table 2). Respiration and utilization sums ranged from 0.00157 ± 0.0002 to 0.109 ± 0.011 μg/L/h. The total utilization (including the intermediates) ranged from 0.0049 ± 0.00059 to 0.0887 ± 0.0089 μg/L/h. Turnover times ranged from about 250 days to almost 30 yr. Recovery for phenanthrene and naphthalene were extremely low, usually less than 3%. These compounds are known to sorb to glass surfaces.

Mineralization rates ranged over four orders of magnitude in the Port Hueneme samples (Table 3), with the most contaminated wells giving the greatest values. The respiration and utilization sums for both benzene and toluene incubations ranged from $9.79 \times 10^{-4} \pm 1.76 \times 10^{-4}$ to 30.5 ± 8.1 μg/L/h. The

Table 2 — Gainesville Sampling, 6 September 1995

Sample	Bacterial Abundance ($\times 10^5$)	Inc. Time (h)	Added ($\mu g \cdot L^{-1}$)	Recov. ($\mu g \cdot L^{-1}$)	Ambient ($\mu g \cdot L^{-1}$)	Respiration + Uptake ($\mu g \cdot L^{-1} \cdot h^{-1}$)	"Total" Util. ($\mu g \cdot L^{-1} \cdot h^{-1}$)	T-over time (days)
Well 3 Phenanthrene	10.4	6:15	59.1	1.19	98	0.0125 ± 0.0044	0.0081 ± 0.003	500
Well 7 Phenanthrene	7.13	5:46	59.1	0.64	29	0.00157 ± 0.0002	0.0049 ± 0.00059	247
UVB Phenanthrene	10.7	6:03	59.1	1.13	13	0.00744 ± 0.0015	0.0166 ± 0.0033	783
Well 7D Naphthalene	4.01	5:41	5.00	0.10	1949	0.09 ± 0.0015	0.00744 ± 0.0015	10915
Well 10 Naphthalene	9.32	5:23	5.00	0.12	1059	0.109 ± 0.011	0.0887 ± 0.0089	497

Table 3 — Port Hueneme Sampling, 17 October 1995

Sample	Bacterial Abundance ($\times 10^5$)	Inc. Time (h)	Added ($\mu g \cdot L^{-1}$)	Recov. ($\mu g \cdot L^{-1}$)	Ambient ($\mu g \cdot L^{-1}$)	Respiration + Uptake ($\mu g \cdot L^{-1} \cdot h^{-1}$)	"Total" Util. ($\mu g \cdot L^{-1} \cdot h^{-1}$)	T-over time (days)
Well 3 Benzene		8:45	6.12	6.25	19387	0.969 ± 0.17	3.13 ± 0.56	258
Well 6 Benzene		8:05	6.12	4.76	18150	1.18 ± 0.14	7.49 ± 0.90	101
Well 7 Benzene		9:05	6.12	4.74	11568	30.5 ± 81	42.7 ± 11	11.0
Well 10 Benzene		7:50	6.12	5.22	15874	0.610 ± 0.05	2.90 ± 0.23	228
UVB Main Benzene		9:45	6.12	1.01	24.2	0.0568 ± 0.003	0.074 ± 0.004	17.0
UVB1 Benzene		9:20	6.12	3.13	81.9	0.0824 ± 0.015	0.165 ± 0.030	22.2
UVB2 Benzene		8:50	6.12	4.06	5.48	0.0175 ± 0.0014	0.021 ± 0.002	23.0
UVB3 Benzene		8:25	6.12	3.78	0.23	0.000979 ± 0.0002	0.00134 ± 0.00024	197
Well 3 Toluene		8:45	6.08	0.92	27362	0.375 ± 0.056	5.19 ± 0.83	220
Well 6 Toluene		8:05	6.08	0.81	30572	0.272 ± 0.033	2.14 ± 0.34	595
Well 7 Toluene		9:05	6.08	0.96	24306	0.704 ± 0.14	1.96 ± 0.39	517
Well 10 Toluene		7:50	6.08	0.89	21338	1.09 ± 0.27	3.84 ± 0.96	232
UVB Main Toluene		9:45	6.08	0.84	25.0	0.0254 ± 0.0055	0.0332 ± 0.0073	39
UVB1 Toluene		9:20	6.08	0.84	23.5	0.011 ± 0.0042	0.0161 ± 0.0045	76
UVB2 Toluene		8:50	6.08	1.25	2.53	0.00682 ± 0.0006	0.0070 ± 0.00063	51.3
UVB3 Toluene		8:25	6.08	0.80	0.22	0.000319 ± 0.0001	0.00144 ± 0.000030	182

total utilization (including the intermediates) ranged from $1.44 \times 10^{-3} \pm 3.02 \times 10^{-4}$ to 42.7 ± 11 μg/L/h. Turnover times ranged from 11 to almost 600 days. The recovery of added isotope averaged 67% for benzene and 15% for toluene. Again, incomplete recovery was likely due to sorption of test substrates to glass surfaces and, potentially, volatilization.

A previously described method [9] called for the use of three additional incubations for cell filtration. We evaluated the effect of acid addition on the cellular fraction by filtering acid-treated and nonacid-treated subsamples. We found no statistical difference ($P < 0.01$) between acid-treated cell fractions and those not treated.

DISCUSSION

A method is described that allows the calculation of mineralization rates for ^{14}C-labeled hydrocarbon growth substrates in environmental water samples. The purpose for the development of updated methods stems from a need to more fully understand the fate of pollutants in the environment at the biochemical level. By the addition of only several simple steps over previously published methodology, the described method allows the investigator to more accurately constrain and interpret recovered $^{14}CO_2$ and cellular fractions from incubated samples. In addition, the investigator is able to quantify the amount of partial biodegradation of tested substrates by analyzing ^{14}C-labeled polar metabolic intermediates. The design

of the method allows for the calculation of an isotope mass balance so that all of the added label can be accounted for. This helps to elucidate sample-to-sample inconsistencies and repetitive pipetting errors. Furthermore, label sorbed to glass surfaces within the incubation vessel or volatilized can be determined, giving a direct measurement of some of the bottle effects common with incubation studies.

Another advantage of the proposed method is greater accuracy in determinating final disintegration-per-minute (DPM) values. Since each fraction is calculated as a percent of the total recovered isotope from the incubation vessel, inconsistencies in pipetting can be minimized. For example, three replicates for $^{14}CO_2$ analysis were 7381, 10138, and 7766 DPM (GCW, Port Hueneme). The standard deviation is 18% of the mean without accounting for the percent of total recovered isotope as would be done if only $^{14}CO_2$ were being assayed. Since this method allows one to determine the fate of all added $^{14}CO_2$, the DPM values are corrected with the mass balance. The standard deviation is only 10% of the mean value. Generally, when substrates are added at tracer concentrations (i.e., 1-5 μL), pipetting errors are more likely to occur.

One last advantage of the described method is the reduction in liquid waste. While use of ^{14}C-labeled substrates (in comparable amounts) will always have a set amount of waste ^{14}C, extraction of unused substrates allows the majority of the isotope used to be confined to a relatively small volume (i.e., 5-mL-per-sample—the extract fraction), while ^{14}C in the majority of the waste (aqueous) is usually only about 1500 DPM/mL.

When the method was applied to environmental samples from Gainesville and Port Hueneme, mineralization rates could be determined. In Gainesville, most rates were at or below the limits of detection. In Port Hueneme, rates were generally higher and had a tremendous range (over four orders of magnitude). Generally, it appeared that rates were higher in areas with higher contamination. This is observed often [10] since bacterial consortia adapted to higher levels generally have increased metabolic capacity. Turnover times were thus fairly comparable, ranging only over an order of magnitude. There are not a large number of field studies with which to compare these data. Although methods varied, a review of PAH biodegradation field data shows PAH half-lives varying greatly (h to yr) [4]. The most comparable data from this report (PAH-exposed ecosystem) shows 14-24 day turnover times for naphthalene and 24-day turnover times for phenanthrene. For BTEX, we compared our results to a report in which groundwater benzene and toluene utilization rates were tested in aquifer microcosms [1] after an acclimation period. Rates reported were several orders of magnitude higher than data obtained in this study, 1040 μg/L/h for benzene (in presence of BTEX) and 208 μg/L/h for toluene (also in the presence of BTEX). We assume that the indigenous consortia in groundwater samples at the contaminated sites were already acclimated to BTEX compounds. Alvarez and Vogel [1] added oxygen to their incubation vessels, whereas we did not. Dissolved oxygen was very low at our field site, so rate differences may be due to its limitation.

Results indicate that in most samples where mineralization is apparent (i.e., production of $^{14}CO_2$), there is a considerable amount of ^{14}C-labeled polar metabolic intermediate production. Although this measurement does not reflect either complete mineralization or cellular incorporation (uptake), it does reflect alteration of the parent compound and thus may help explain some of the differences usually seen between chemical analysis of product disappearance, bulk CO_2 production, and production of $^{14}CO_2$ during biodegradation assays [10]. Since a mass balance is calculated, the fate of all fractions of added ^{14}C-labeled test substrates can be determined.

The described method produces interesting results when investigating the use of extremely nonpolar substrates, such as PAHs. Calculations of isotope recovery for naphthalene and phenanthrene indicate that most of the label is retained on glass surfaces after incubation. This is not entirely surprising since the

aqueous solubility of PAH compounds is extremely low [7]. It is likely that groundwater bacteria, particularly those from a contaminated well site, are able to attach to surfaces containing growth substrates [8]. If this is the case, one would expect to find radio label incorporated into cells preferentially attached to the surface wall of the incubation vessel. It is interesting to note that in several instances (wells 3, 7, and GCW at Gainesville), positive values for respiration were obtained, yet values for cellular incorporation were negative. If bacteria are preferentially attached to the PAH/glass surface, incorporated label would not appear in the cellular fraction nor would label that was passively adsorbed to those bacterial surfaces. Therefore, the control vessels might have more cellular label solely because there are more cells (i.e., not able to attach to PAH/glass surfaces because formalin added at $t = 0$).

A method is described that refines previous protocols to provide a relatively simple, rapid assay of the bioremediation of selected pollutant substrates. The advantage of this method is the ability to calculate a mass balance for added isotope by collecting different fractions of the test sample. Relation of these fractions to different steps in the biodegradation of test compounds provides the investigator with a more definitive measure of utilization kinetics. Other advantages include increased accuracy and precision and a decrease in the volume of radioactive waste produced. The method requires slightly more time and resources than traditional techniques, however the information gained will allow more accurate estimations of biodegradation rates in field settings and ultimately lead to a better understanding of field bioremediation.

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